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Complications in the study of ancient tuberculosis: Presence of environmental bacteria in human archaeological remains

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ABSTRACT

There are many reports of ancient DNA from bacteria of the *Mycobacterium tuberculosis* complex (MTBC) being present in skeletons with and without osteological indications of tuberculosis. A possible complication in these studies is that extracts might also contain DNA from the microbiome of the individual whose remains are being analysed and/or from environmental bacteria that have colonised the skeleton after death. These contaminants might include 'mycobacteria other than tuberculosis' (MOTT), which are common in the environment, but which are not normally associated with clinical cases of tuberculosis. In this paper we show that MOTT of various types, as well as bacteria of related genera, are present in most if not all archaeological remains. Our results emphasise the complications inherent in the biomolecular study of archaeological human tuberculosis. The specificity of any polymerase chain reaction directed at the MTBC cannot be assumed and, to confirm that an amplification is authentic, a sequencing strategy must be applied that allows characterisation of the PCR product. Any variations from the reference MTBC sequence must then be checked against sequence data for MOTT and other species to ensure that the product does actually derive from MTBC. Our results also illustrate the challenges faced when assembling MTBC genome sequences from ancient DNA samples, as misidentification of MOTT sequence reads as MTBC would lead to errors in the assembly. Identifying such errors would be particularly difficult, if not impossible, if the MOTT DNA content is greater than that of the authentic MTBC. The difficulty in identifying and excluding MOTT sequences is exacerbated by the fact that many MOTT are still uncharacterized and hence their sequence features are unknown.

1. Introduction

Tuberculosis (TB) is caused by members of the *Mycobacterium tuberculosis* complex (MTBC) of bacteria. These species include *M. tuberculosis*, which is the most frequent cause of human TB, the less common human pathogens *Mycobacterium africanum* and *Mycobacterium canettii*, as well as *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium pinnipedii* and *Mycobacterium caprae*, which are primarily responsible for TB in animals but have also been known to infect humans (Gutiérrez et al., 1997; Van Soolingen et al., 1997, 1998; Aranaz et al., 2003; Kiers et al., 2008). Although principally a pulmonary disease, 3–5% of modern-day untreated TB patients develop bone changes following spread of bacteria through the body via the blood and lymphatic systems (Jaffe, 1972). Typical lesions include collapse of the lower thoracic and upper lumbar vertebrae resulting in a curvature of the spine, called Pott's disease, and damage to major joints, particularly the hip or knee (Resnick, 2002). More non-specific bone changes have also been identified as potentially related to TB in archaeological skeletons, such as granular impressions and new bone formation on the endocranial surface of the skull, new bone formation on the visceral surfaces of the ribs, hypertrophic pulmonary osteoarthropathy and dactylitis (see Roberts and Buikstra, 2003:99–109 for a summary). These non-specific lesions can be recognized in archaeological skeletons, and although not specific for TB provide indications of the possible prevalence of the disease in past societies.

The excavation of skeletons with indications of TB, and the likely presence of MTBC bacteria in the bones at time of death, has made TB an attractive target for ancient DNA (aDNA) studies. The first report of *M. tuberculosis* aDNA detection (Spigelman and Lemma,

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1993) has been followed by a substantial number of publications describing the use of the polymerase chain reaction (PCR) to detect MTBC in human bones and teeth (e.g. Baron et al., 1996; Taylor et al., 1996, 1999; Faerman et al., 1997; Haas et al., 2000; Mays et al., 2001, 2002; Zink et al., 2001, 2003, 2005; Mays and Taylor, 2003; Donoghue et al., 2005; Müller et al., 2014a, 2014b), mummified soft tissue (e.g. Salo et al., 1994; Nerlich et al., 1997) and calcified pleura (e.g. Donoghue et al., 1998), spanning a time frame from as early as 9000 BC (Hershkovitz et al., 2008) through the Iron Age (e.g. Mays and Taylor, 2003) and the Medieval periods (e.g. Faerman et al., 1997) and up to modern times (e.g. Zink et al., 2005). More recently, next generation sequencing (NGS) methods, with or without pre-capture of MTBC aDNA, have been used to obtain genomic data from skeletal and mummified human remains (Bouwman et al., 2012; Chan et al., 2013; Bos et al., 2014).

A possible complication in the study of samples for MTBC aDNA is the presence of DNA from the microbiome of the individual whose remains are being analysed and/or from environmental bacteria that have colonised the skeleton after death (Wilbur et al., 2009; Tsangaras and Greenwood, 2012; Müller et al., 2015). These contaminants might include 'mycobacteria other than tuberculosis' (MOTT), which are common in the environment and may occasionally cause opportunistic disease, but which are not normally associated with clinical cases of TB. The genus *Mycobacterium* is often classified into two major groups, slowly and rapidly growing mycobacteria, a phenotypical division which appears to be supported by genomic data (e.g. Khan and Yadav, 2004; Kweon et al., 2015; Wang et al., 2015). The former group comprises the MTBC and other opportunistic pathogens (human or animal), whereas fast-growing mycobateria are predominantly non-pathogenic (Wang et al., 2015). The PCRs used to detect MTBC are generally considered to be specific when used with clinical samples, but most clinical samples do not contain extensive MOTT contamination. False-positives might therefore occur when they are used with more complex extracts, such as those from skeletons that have been buried in the ground for centuries (Wilbur et al., 2009). We have recently shown that this is the case with the standard PCR used to detect the IS6110 sequence, which was previously thought to give positive results only with DNA from the MTBC, but which also amplifies fragments of the same length from environmental bacteria that are present in at least some human archaeological skeletons (Müller et al., 2015). Contamination with MOTT species might also complicate assembly of MTBC genome sequences after NGS of archaeological bone or dental samples, if sequence reads derived from environmental species are mistaken for ones representing an MTBC member (Bouwman et al., 2012).

In this paper we assess the extent and nature of the MOTT and broader bacterial content of human archaeological skeletons, including several displaying potential osteological indicators of TB. We present the results of two independent analyses. First, we report the identities of MOTT present in different skeletons and show the taxonomic relationships of these with MTBC. Second, we provide examples of the non-specificity of PCRs directed at the MTBC, and describe the contaminating sequences and their likely derivation.

2. Materials and methods

2.1. Samples

We studied 22 samples taken from bones or teeth from 21 skeletons from 1st–19th centuries AD from 13 sites in Britain and three from continental Europe (Table 1). Fourteen of these skeletons displayed pathological alterations possibly suggesting infection with MTBC. In previous work (Müller et al., 2014a) we have obtained reproducible IS6110 PCR products from 11 of these samples, checked by sequencing of the cloned amplicons, and less reproducible results for IS6110 and/or a second insertion element, IS1081 with seven other samples. The remaining four samples gave no evidence for the presence of MTBC aDNA (Table 1). Most of the samples were from individuals buried in earth-cut graves, with direct exposure to the surrounding soil, but three individuals were recovered from vaults (St George's Crypt 4006, St George's Crypt 5003, St Peter's Collegiate Church 62).

Precautions used to prevent contamination of samples with exogenous DNA, and the methods used to prepare DNA extracts, are described in Müller et al. (2015).

2.2. Polymerase chain reactions

Polymerase chain reactions were set up in 30 μl reactions containing 2.5–5.0 μl DNA extract, 1 × AmpliTaq Gold PCR Master Mix (Applied Biosystems), 400 nM each primer, 1% BSA and ultrapure water. PCRs were run for one cycle for 7 min at 95 °C, followed by 40–45 cycles each consisting of 1 min at the annealing temperature, 1 min at 72 °C and 1 min at 95 °C, with a final extension for 10 min at 72 °C. Six genetic loci were studied (Table 2) using the primer sequences and PCR conditions given in Table 3. Amplification products were run on a 2% agarose gel and purified products cloned into *Escherichia coli* XL1-Blue competent cells (Agilent) using the CloneJet PCR cloning kit (Thermo Scientific). Clones were sequenced (GATC Biotech, Cologne) and aligned with the respective reference sequences using Geneious version 7.1.7 (available from http://www.geneious.com/). BLAST (Altschul et al., 1990) was used to compare sequences with the GenBank nucleotide database. For hsp65, 187 bp fragments obtained after primer removal were also aligned to the homologous regions of 168 mycobacteria from the 'List of Prokaryotic names with Standing in Nomenclature' (Euzéby, 1997). A maximum likelihood (ML) tree was created using Mega version 6 (Tamura et al., 2013). The Jukes-Cantor model for nucleotide substitution (Jukes and Cantor, 1969) was applied and 1000 bootstrap replicates were used to determine the robustness of the tree topology. The tree was visualized with FigTree version 1.4.2 (available from http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

3.1. PCRs directed at the mycobacterial heat-shock protein gene hsp65

We applied a genus-specific PCR directed at the heat shock protein gene hsp65 (Khan and Yadav, 2004) to ten samples, cloned the PCR products, and sequenced 2–6 clones per sample in order to identify the mycobacterial species that were present. None of the sequences exactly matched any of the mycobacterial hsp65 entries contained in GenBank, but BLAST searches identified all sequences as most likely deriving from MOTT species. Only one sequence type was obtained for each of the individuals from Ashchurch 705, Horncastle 20, Kempston 3902 and Obeliai 143A, but all the other samples displayed at least two different sequence types (Supplementary Fig. 1).

A maximum likelihood tree constructed from the clone sequences and the equivalent hsp65 regions of 168 mycobacterial species displayed the expected division of *Mycobacterium* into slow and rapid growers, with the exception of a few species which did not fall into their respective clusters (Fig. 1). The tree suggests that the sequences obtained from individuals Ashchurch 705 and Horncastle 20 fall within the group of slow-growing mycobacteria, and those from Kempston 3902, Obeliai 143 and Queensford Mill